A FLUOROMETRIC METHOD FOR THE ESTIMATION OF TRYPTOPHAN*

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The various colorimetric methods now employed for the estimation of tryptophan are not specific for tryptophan but give colored products with many compounds containing the indole nucleus, including indole itself. In order to facilitate studies of the enzymatic synthesis of tryptophan from indole and serine by extracts of *Neurospora* (1), a search for a rapid, quantitative method for the estimation of tryptophan in the presence of indole was undertaken. Tauber (2) reported that tryptophan gives a green fluorescence when treated with 70 to 72 per cent perchloric acid at room temperature. Modification of this procedure has led to a method for the rapid estimation of tryptophan, without preliminary extraction of indole, in hydrolyzed or unhydrolyzed tryptophan-containing materials.

EXPERIMENTAL

Apparatus—Intensity of fluorescence is measured with a Coleman electronic photofluorometer, model 12B. The light entering from the mercury vapor lamp is caused to pass through a PC-3 filter and the resulting fluorescence through a PC-10 filter. A $\frac{3}{4}$ in. round test-tube of the type ordinarily used with this instrument is fitted with a leucite ring bored to support a 22 × 100 mm. test-tube inside the larger tube. Test solutions are placed in the inner tube. A volume of 2.5 ml. is sufficient to allow the entering light to focus in the test solution. Under these conditions readings are reproducible to within ±2 scale divisions.

Reagents—

1. 70 to 72 per cent perchloric acid (Merck reagent).
2. Aqueous indole. Dissolve 117 mg. of indole (Eastman) in 100 ml. of distilled water.
3. Aqueous γ-(indole-3)-*n*-butyric acid. Dissolve 10 mg. of γ-(indole-3)-*n*-butyric acid (Eastman) in 100 ml. of distilled water.

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4. 10 per cent acid-hydrolyzed casein (Nutritional Biochemicals Corporation).

5. Phosphate buffer, pH 7.5 to 8.0. Dissolve 14.7 gm. of disodium hydrogen phosphate (Merck reagent) in distilled water and make up to a liter. Adjust pH with phosphoric acid.

6. Supplement. Mix 1 ml. of indole solution, 1 ml. of γ-(indole-3)-*n*-butyric acid solution, and 1 ml. of 10 per cent acid-hydrolyzed casein. Dilute to 10 ml. with phosphate buffer. The supplement should be made up fresh weekly and kept in a refrigerator when not in use.

Procedure for Assay—In order to obtain results which have quantitative significance it is necessary to stabilize the fluorescence. Such stabilization can be effected by the use of the supplement described above. The sample to be tested is taken up in the phosphate buffer and 0.4 ml.

TABLE I

Fluorescence Obtained by Treating Certain Indole Derivatives with 70 to 72 Per Cent Perchloric Acid

Each tube contains 0.05 μM of the compound.

Compound	Scale reading	Compound	Scale reading
Indole*	2	β-(Indole-3)-*n*-propionic acid	70
Indole-2-carboxylic acid	1	γ-(Indole-3)-*n*-butyric acid	35
Indole-3-carboxylic acid	2	Tryptophan*	100
3-Methylindole	38		
Indole-3-acetic acid	55		

*The photofluorometer is adjusted to a zero scale reading with a mixture of 0.5 ml. of distilled water and 2 ml. of 70 to 72 per cent perchloric acid. The sensitivity is so adjusted that the tryptophan tube reads 100.

is added to 0.1 ml. of the supplement solution. 2 ml. of perchloric acid are added rapidly at room temperature, the solution is well mixed, warmed for 1 hour at 40°, and allowed to cool to room temperature. A series of tubes prepared in the same manner containing 0, 2, 4, 6, and 8 γ of tryptophan is used to adjust the photofluorometer. The instrument is adjusted to a zero scale reading with the blank tube and the sensitivity is adjusted with the 8 γ tube to a scale reading of 100. The intermediate tubes will read approximately 25, 50, and 75, respectively. The unknown solutions are read with this setting of the instrument. Occasional readjustments of the zero and 100 readings are desirable during the course of a large number of determinations. Since the intensity of fluorescence slowly diminishes, it is necessary to adjust the instrument with a control series prepared at the same time as the unknown solutions. The linear relationship between the intensity of fluorescence and the concentration of tryptophan persists for about 24 hours.

Influence of Various Materials on Relative Stability of Fluorescence—The relative stability of fluorescence is a sensitive function of several materials which do not contain tryptophan. High concentrations of many inorganic

TABLE II

Tryptophan Found in Various Materials (in Per Cent Tryptophan)

Material	Unhydrolyzed*	Hydrolyzed	Literature values
Albumin egg scales†	2.04	1.88	
	1.98	1.96	
	2.04		
Crystalline bovine serum albumin‡	0.72	0.68	0.57 (4)
	0.76	0.70	0.65 (5)
	0.69	0.65	0.68 (5)
Casein§	1.40	1.33	1.32 (4)
	1.32	1.27	1.38 (5)
	1.38	1.24	1.54 (6)
Edestin‖	1.44	1.30	1.3 (7)
	1.46	1.29	1.3 (8)
	1.38	1.32	1.2 (4)
			1.4 (10)
Gelatin¶	0.016	0.010	0.0 (11)
	0.021	0.012	0.0 (9)
	0.017		0.1 (12)
Gluten**	0.84	0.75	0.91 (4)
	0.86	0.81	0.93 (6)
	0.94		1.2 (13)
Zein††	0.22	0.21	0.2 (14)
	0.20	0.19	0.2 (9)
			0.0 (12)
Hot water *Neurospora* extract (autolyzed)	0.34	0.30	
	0.32	0.29	
Acid-hydrolyzed casein + 2% added tryptophan	2.00	1.89	
	2.08	1.84	
	2.04	1.90	

* Separate determinations on the same sample.
† Albumin egg scales, J. T. Baker Chemical Company.
‡ Crystallined bovine serum albumin, the Armour Laboratories, G 4502 plasma.
§ Casein, vitamin-free S. M. A. Corporation batch No. 12537.
‖ Edestin, laboratory sample of unknown origin.
¶ Gelatin, Difco batch No. 326614.
** Gluten, Braun, Knecht, Heimann Company.
†† Zein (corn), Pfanstiehl Chemical Company, batch No. 536.

ions, concentrations of organic materials above 10 mg. per tube, prolonged heating or exposure to direct sunlight or ultraviolet light, and either too high or too low a concentration of hydrogen ion cause uneven fading of fluorescence. The Na_2HPO_4 adjusts for this last effect, except in strongly

alkaline or acidic solutions. In such cases the sample should be neutralized before being taken up in the buffer solution.

Occasional samples of perchloric acid, when mixed with tryptophan by the described procedure, will not produce the substances which fluoresce under ultraviolet light. It seems probable that these samples are contaminated with one or more agents which inhibit fluorescence.

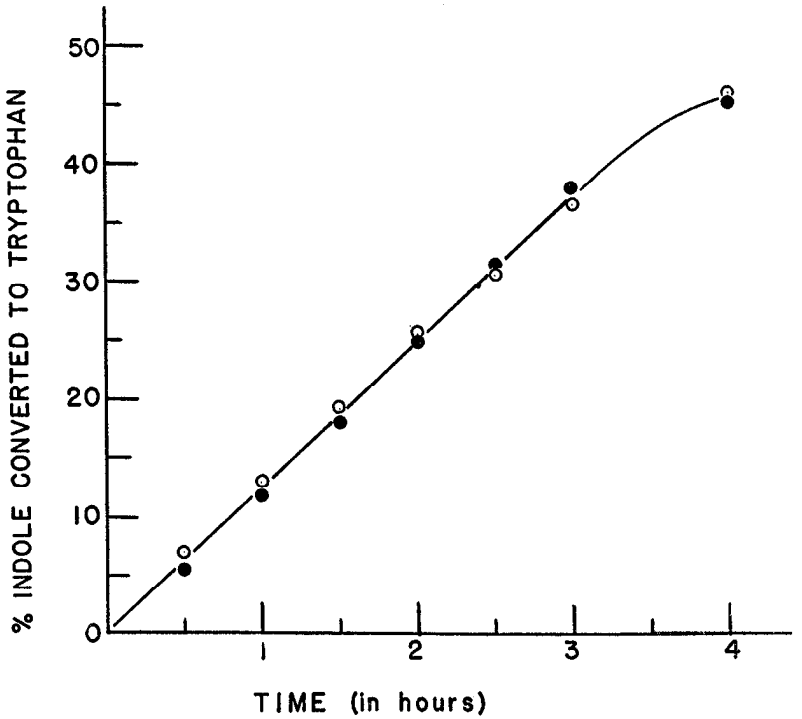

FIG. 1. Per cent conversion of indole to tryptophan by extracts of *Neurospora*. ○, calculated from tryptophan found by perchloric acid method; ●, calculated from indole found by xanthydrol method of Fearon.

Specificity of Test—Table I gives data comparing the fluorescence obtained when 0.05 μM of several compounds containing an indole nucleus is treated with 70 to 72 per cent perchloric acid. Tests for tryptophan based upon its reaction with aldehydes in the presence of mineral acids are thought to be characteristic of indole and indole derivatives containing a hydrogen-substituted 2-carbon atom. In contrast, the fluorescence obtained in this test appears to be a function of both a hydrogen-substituted 2-carbon atom and a side chain in the 3 position of the indole nucleus.

Estimation of Tryptophan in Various Organic Materials—Table II gives

tryptophan values obtained by the above procedure for various substances both before and after hydrolysis for 1 hour with 2 N NaOH. All samples were dried *in vacuo* for 4 hours at 65° over P_2O_5. Literature values for tryptophan in similar materials are listed for comparison.

Fig. 1 is a plot of the increase of tryptophan concentration found by this method when a crude dialyzed *Neurospora* extract is treated with pyridoxal phosphate, indole, and serine, and incubated at 35° at pH 7.8. On the same graph is plotted the percentage conversion of indole to tryptophan obtained by measuring the decrease in indole concentration by the method of Fearon (3).

DISCUSSION

The nature of the reactions involved in the method herein described is unknown. Therefore, justification of the use of the recommended mixtures of amino acids, indole, and γ-(indole-3)-*n*-butyric acid to obtain relative stability of fluorescence must be a strictly empirical one. However, other workers have recommended the use of amino acid mixtures to stabilize tryptophan in highly acidic solutions (4, 15, 16). Graham *et al.* (4) suggest the use of 35 mg. of gelatin in order to stabilize the color obtained when tryptophan is estimated by the Bates (17) procedure. Since the samples of gelatin tested during this investigation contained detectable traces of tryptophan, attempts were made to obtain relative stability of fluorescence by use of acid-hydrolyzed casein. All experiments with this in view were negative and the addition of an indole-containing compound with a side chain in the 3 position was required before adequate stability was obtained. The test was standardized with γ-(indole-3)-*n*-butyric acid because of the low fluorescence produced by it in this procedure. Although the test could no doubt be modified by using small amounts of tryptophan itself as the stabilizing indole component, it seems less ambiguous to use a material which is unknown in biological systems.

Readings on solutions containing 1 to 8 γ of tryptophan can be reproduced to within ±2 scale divisions. However, with scale readings below 50, this represents an error of at least 4 per cent, depending upon where the reading falls on the scale. If the samples to be tested are chosen so that they contain 6 to 8 γ of tryptophan, determinations can be repeated with an error of not more than ±5 per cent.

Tauber (18) has reported that small quantities of dichromate enhance the fluorescence obtained in his qualitative test. With the quantities of tryptophan used in this work, dichromate was found to have a deleterious effect on the stability of the fluorescence.

SUMMARY

A new method for the estimation of tryptophan has been described. Tryptophan can be quantitatively determined in unhydrolyzed proteins. Determinations are reproducible approximately to within 5 per cent.

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